Stress treatments and ficoll for improving green plant regeneration in triticale anther culture

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Abstract

Anther culture of 10 winter triticale cultivars of diverse origin was studied. Two-week cold pretreatment was applied to all cultivars. In addition, cold pretreatment in combination with heat shock or mannitol starvation and prolonged cold stress of three weeks were tested on some of the genotypes. Field and greenhouse conditions were compared for growing the mother plants. Anthers for stress treatment testing were cultured on solid W14. Ficoll-supplemented induction medium was tested with three cultivars. A total of 378 green regenerants were analysed for DNA-ploidy with flow-cytometry. Morphological observations and seed-set were recorded for 317 plants grown to maturity. Large genotypic differences were noted between the cultivars. Seven out of 10 cultivars exhibited a differential response to the treatments and it was possible to improve green plant production considerably by applying increased stress or by culture on a medium containing ficoll. Cold pretreatment complemented with heat shock improved androgenetic response of the three cultivars tested. Prolonged cold pretreatment increased generally both the proportion of green plants and the proportion of spontaneous doubling. Morphological abnormalities, possibly caused by aneuploidy and associated with loss of fertility, were observed in 23% of the plants. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: × Triticosecale; Stress treatment; Doubled haploid; Green plant regeneration; Plant morphology; Spontaneous doubling

1. Introduction

Triticale (× Triticosecale Wittmack) can be considered to be a self-pollinated cereal, for which doubled haploids (DHs) can be used effectively as a tool in breeding. DHs permit screening of qualitative characters due to inherent homozygosity. Increased selection efficiency of quantitative characters follows from absence of dominance genetic variation and epistatic effects [1]. DHs can also be useful in triticale breeding for fixing homozygosity after interspecific and intergeneric hybridization. This represents one possible strategy for improving local adaptation of triticale lines in, for instance, environments characterised by a requirement for good winter hardiness. Triticale is a potential winter cereal for northern latitudes. Adapted local rye and wheat germplasm could be used in triticale breeding programmes for such areas.

The use of DHs in triticale breeding programmes is limited by the relatively low cost-efficiency of anther culture [2]. In anther culture systems stress is of critical importance for blocking gametophytic development and for triggering pollen embryogenesis in competent microspores [3]. Other means for improving the yield of microspore-derived green plants of cereals have included culture in liquid medium supplemented with a buoyancy increasing component, ficoll (type 400), to allow anthers and calli to float [4,5]. With wheat, Triticum aestivum L. [6] and rye, Secale cereale L. (S. Immonen, unpublished data), the results have, however, been inconsistent.

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In these experiments, we tested 10 winter triticale cultivars that had not previously been tested for their anther culture ability. The selected cultivars represented the genetic diversity of triticales incorporated into the Finnish winter triticale breeding programme. The aims were to test various stress treatments and culture media to improve the in vitro performance of triticale, to evaluate this diverse material for use in DH-production and to obtain information on the quality of the regenerants.

2. Materials and methods

2.1. Plant material

The cultivars included ‘Ulrika’ (Svalöf-Weibull), ‘Asmus’ (Nordsaat), LP 4496.5.92 (Lochow-Petkus), ‘OAC Trillium’ (Parsons Seeds, Canada), ‘OAC Wintri’ and ‘Pika’ (Alberta Field Crop Development Centre), ‘Paljus’ and ‘Modul’ (Byelorussian Scientific Research Institute of Arable Farming), Bor 96151 and Bor 96152 (Boreal Plant Breeding). The mother plants were grown in the greenhouse and were vernalised during winter and grown under a 20°C:15°C day:night temperature regime with at least a 16 h natural light photoperiod, supplemented with fluorescent lamps. The lines ‘Asmus’, ‘Paljus’, LP 4496.5.92, ‘Trillium’, ‘Ulrika’ and ‘Modul’ were also grown in the field.

2.2. Anther culture

Spikes were collected when microspores were at the mid to late uninucleate stage. The tillers were cut and all leaves were removed except the flag leaf. The tillers were then covered with a plastic bag and stored at 4°C with stalks in water for 2 weeks unless otherwise stated. Prior to excision of the anthers, the developmental stage of the microspores was determined by squashing the anther in acetocarmine and studying the microspores under the light microscope. The spikes were surface sterilized by agitating them for 20 min in 1.2% sodium hypochlorite solution with 0.5% polyoxyethyleneorbitan monooleate (Tween 80), followed by thorough rinsing in sterilised water. The induction medium consisted of W14 macro and micro elements [7], MS vitamins and iron [8], 9 μM 2,4-dichlorophenoxyacetic acid, 2.3 μM kinetin, 9% maltose and Phytagel™ (Sigma) at 3%. The cultures were incubated at 25°C in the dark for 8–10 weeks. Embryogenic structures were transferred to regeneration medium consisting of half strength MS salts, MS iron and vitamins, 2.9 μM indole-3-acetic acid, 4.6 μM kinetin, 3% sucrose and 3% Phytagel. All media were adjusted to pH 5.8 prior to autoclave sterilisation (15 min, 121°C, 20 psi). Subcultures were incubated at 21°C in a 16-h light, 8-h dark photoperiod for 5 weeks, after which green plants were potted in a peat-soil mixture, vernalised during winter and grown to maturity during the following season. A total of 378 green regenerants were analysed with flow-cytometry at the plantlet stage as described by Immonen et al. [9].

2.3. Experiments

All 10 cultivars were pretreated at +4°C for 2 weeks (control treatment) and all but ‘OAC Trillium’ also for 3 weeks. In addition, sets of three arbitrarily chosen cultivars were given one of the following treatments: post-plating heat shock at +32°C for 3 days, culture on medium containing 175.6 mM mannitol for starvation treatment at +4°C, culture on liquid medium supplemented with 100 g l⁻¹ Ficoll® 400 (Pharmacia Biotech). The cultivars grown in the field were treated in a similar way as the control. Cultivars for the experiments were chosen arbitrarily as no information was available on their responsiveness in anther culture.

2.4. Observations and statistical analysis

The numbers of transferred calli and green and albino plants were recorded. Small plantlets with roots were also included in the count, irrespective of whether they survived transfer to soil or not. The impact of each treatment was assessed in comparison with the control for those cultivars included. Thus the treatments tested on different cultivars could not be directly compared. The following parameters were used: induction (percentage calli from plated anthers), green plant production (percentage green plants from plated anthers), regeneration efficiency (green plants per transferred calli), and ratio of green to albino plants. The morphology of the plants was ob-
served and two categories of abnormal morphology were established: grass-like and stunted. Seedset was recorded. Mixed model analysis was applied to the induction data [10]. Frequency data on green plant regeneration efficiency, green to albino plant ratios and ploidy were analysed using the \( \chi^2 \)-test.

3. Results

3.1. Treatment effects on anther culture response

Large differences between the cultivars were detected in anther culture induction in the control treatment of 2 weeks cold stress, which was applied to all cultivars (Table 1). The best and poorest genotypes ranked similarly also under the other stress treatments: ‘Ulrika’, ‘Pika’ and ‘OAC Trillium’ were consistently the most recalcitrant and ‘OAC Wintri’ showed by far the best induction capacity. Cultivars also differed in their regeneration efficiency, which was not correlated with the induction response. Bor 96151 had a consistently high regeneration efficiency resulting in the highest green plant production, while ‘Wintri’ had a relatively low regeneration efficiency.

We observed statistically significant treatment \( \times \) genotype interactions for induction. Growth of mother plants in the field significantly enhanced induction (\( P < 0.0001 \)) for ‘Asmus’ and reduced it (\( P = 0.0021 \)) for ‘Modul’ in comparison with the growth in the greenhouse. Prolonged cold pretreatment of greenhouse grown tillers was significantly better (\( P = 0.0242 \)) for induction in comparison with the field conditions over all the cultivars tested. Among greenhouse grown material a differential induction response to the length of cold pretreatment was recorded for the cultivars, and prolonged cold was significantly better (\( P < 0.0001 \)) only for ‘Asmus’ (Table 1). A 3-week cold pretreatment increased green plant regeneration efficiency significantly with ‘Paljus’ (\( P < 0.001 \)), ‘Asmus’ (\( P < 0.001 \)), ‘Ulrika’ (\( P < 0.001 \)), Bor 96152 (\( P < 0.05 \)) and ‘Modul’ (\( P < 0.001 \)) in comparison with 2 weeks. For the other cultivars the differences in regeneration efficiency were not statistically significant. Prolonged cold pretreatment enhanced the green to albino plant ratio for seven out of nine cultivars in comparison with 2 weeks of cold (Table 2). For ‘Asmus’ and ‘Modul’ this increase was significant (\( P < 0.001 \)). For ‘OAC Wintri’ prolonged cold pretreatment increased albino plant regeneration in particular (Table 3).

The effect of mannitol stress depended on the cultivar. With Bor 96152 mannitol stress increased induction significantly (\( P < 0.0001 \)) in comparison with cold stress of either length, but green plant regeneration efficiency was significantly lower than after 3 weeks of cold pretreatment. ‘Asmus’ re-

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Field</th>
<th>Greenhouse</th>
<th>Ficoll</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Solid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 week</td>
<td>2 week</td>
<td>3 week</td>
<td>2 week + mann.</td>
</tr>
<tr>
<td></td>
<td>CA ± S.E.</td>
<td>CA ± S.E.</td>
<td>CA ± S.E.</td>
</tr>
<tr>
<td>Bor96151</td>
<td>13.6 ± 2.1bed</td>
<td>8.9 ± 2.2def</td>
<td>16.3</td>
</tr>
<tr>
<td><code>Ulrika</code></td>
<td>8.3 ± 1.2c</td>
<td>9.0 ± 1.1de</td>
<td>4.0 ± 0.8</td>
</tr>
<tr>
<td><code>OAC Trillium</code></td>
<td>6.0 ± 1.3c</td>
<td>7.4 ± 1.4de</td>
<td>14.8 ± 2.0</td>
</tr>
<tr>
<td><code>Pika</code></td>
<td>2.4 ± 0.7f</td>
<td>2.4 ± 0.7f</td>
<td></td>
</tr>
</tbody>
</table>

\( a \) CA, percentage of anthers plated; \( n, 800 \), except for 3-week stress treatment for which \( n \) varies from 250 to 800; numbers in columns with the same letter are not significantly different (\( \chi^2, P > 0.05 \)).

\( b \) Cold pretreatment of tillers combined with mannitol starvation or heat as indicated.

\( c \) Based on sums.
sponded in a similar way to mannitol and cold treatments. For ‘Ulrika’ 3-week cold pretreatment was superior to mannitol stress in regeneration efficiency. Heat stress increased induction significantly ($P < 0.05$) with all cultivars tested (Table 1). For ‘Paljus’ and LP 4496.5.92 the increase was, however, not significantly different from that of the 3-week cold pretreatment. Regeneration efficiency of the heat stressed cultures was also relatively high, resulting in high total plant yields and numbers of green plants, particularly with the cultivars ‘Paljus’ and LP 4496.5.92 (Tables 2 and 3). Ficoll in liquid medium promoted a three- to fourfold increase in induction for Bor 96151 in comparison with the solid medium. For the two other cultivars cultured on ficoll medium, ‘Modul’ and ‘Wintri’, induction either remained the same or decreased (Table 1). For ‘Wintri’ ficoll promoted significantly better regeneration efficiency ($P < 0.0001$) than solid medium.

### 3.2. Quality of regenerated plants

Bor 96151, Bor 96152 and ‘Paljus’ exhibited a consistent green to albino plant ratio ($P > 0.05$) irrespective of the treatment applied (Table 3). Bor96151 yielded 84.3% green plants on average. Prolonged cold pretreatment was associated with a relatively high proportion of green plants with all other cultivars, except ‘OAC Wintri’. Comparison of the treatments in more detail was not possible due to the large differences between cultivars in absolute numbers of green plants produced and as proportions of all regenerated plants.

Spontaneous doubling of the regenerants deriving from the different tests was discussed by Immonen et al. [9] after flow-cytometric analysis of the plantlets. The pooled data including all treatments are shown in Table 4. Statistical analysis indicated three levels of spontaneous doubling. In pairwise comparison of the treatments, 3-week cold pretreatment increased spontaneous doubling significantly ($P < 0.05$) in comparison with 2-week cold pretreatment of either greenhouse grown material (44°C:57°C vs 20°C:51°C) or field grown material (39°C:45°C vs 5°C:22°C) and heat shock treatment (23°C:11°C vs 13°C:34°C). Limited seed-set was noted also in some plants initially classified as haploids (Table 5).

Numbers of green plants from all experiments were pooled for analysis of morphological observations. Distinct morphological changes were observed and the plants were classified as normal, stunted or grass-like (Table 5). The stunted character was associated with a club-like spike and very low fertility. On average, DNA-content in that category was considerably reduced both among the 2C and C plants. Root-tip analysis revealed a loss of one or two chromosomes in some cases. The 2C plants in the grass-like category had reduced fertility and DNA-content in comparison with the normal plants (Table 5).
4. Discussion

Most of the 10 triticales, which came from very different breeding programmes, showed relatively good androgenetic response in terms of green plant production. Only ‘Pika’ remained below the 1% level, which was considered to be a good anther culture response by Stober and Hess [11]. Bor 96152 and ‘OAC Trillium’ did not show improved response to any of the treatments applied to them. For the remaining cultivars large differences were detected between treatments and considerable improvement in green plant production was possible by altering the stress or medium condition. Green plant production was 3.3% on average and, combining the best result for each cultivar, reached 6.1%. With triticale there is obviously genotypic potential for considerable enhancement in androgenetic green plant production although the optimum protocol may depend on the genotype. In general, ficoll treatment, heat shock and prolonged cold pretreatment improved green plant production in comparison with control and mannitol starvation. In these experiments better results were obtained from greenhouse grown plants than from those grown in the field.

Green plant production involves three independent phenomena: induction, regeneration efficiency of the embryoids and the production of green plants, as discussed by Gonzáles et al. [12]. ‘Wintri’ exhibited a negative correlation between induction and regeneration efficiency. Such differential response was also observed in some cases with ‘Ulrika’ and Bor 96151. There was evidence of genotypic determination for a favourable green to albino plant ratio. The average proportion of green plants over all cultivars and treatments was 57%. The highest ranking cultivar, Bor 96151, showed a constant ratio of green to albino plants irrespective of the treatment (84.3%, 241:45). The poorest cultivars in this regard, ‘Wintri’ and Bor 96152, yielded 34.1 and 24.5% of green plants respectively. Tuvesson et al. [13] and Olesen et al. [14] showed that nuclear genes affected the frequencies of chloroplast genome aberrations causing albinism. Various environmental factors have been shown to influence albinism. With this material cold pretreatment improved the green plant ratio in general, in contrast to the findings of Barceló et al. [15] with tritordeum. Both the green plant yields and the green plant ratios were relatively high in these experiments in comparison with previous reports for triticale. Gonzáles et al. [12] reached 1% green plant regeneration on average using sucrose for induction and Marciniak et al. [16] reported the best green plant production (2.3%) from medium containing maltose. Karsai and Bedő [17] reported high green plant yield (> 10%) for ‘Monico’ on induction medium with the same maltose concentration as used here. Bernard et al. [2] described the use of DHs in triticale breeding listing a typical response for randomly selected triticale crosses. The average green plant yield was 1.6%, ranging from 0.1 to 6.5%. The average proportion of green plants was 55% and the best cross yielded 82% green plants,
which corresponds well with our results. In contrast with the findings of Tuvesson et al. [13] for wheat, regeneration ability and proportion of green plants were not correlated here nor were they for the material of Bernard et al. [2], although the lines with the best green to albino plant ratio also showed high regeneration ability.

External stress is important for directing the microspores to undergo embryogenesis. With rye, 3–4 weeks of cold pretreatment elicited substantial production of green plants of recalcitrant cultivars, but mannitol starvation of untreated anthers suppressed induction [18]. A combination of heat shock and starvation promoted embryogenesis in high frequencies with recalcitrant wheat lines [19]. Here we tested cold pretreatment in combination with heat shock or mannitol starvation. The responses were, to some extent, genotype-specific. The results indicate that adequate cold pretreatment alone, or in combination with heat shock could be applied to a broad range of triticale genotypes to promote androgenetic green plant production. Heat shock did not have an adverse effect on any of the genotypes tested. Lukjanjuk and Ignatova [20] reported that cold pretreatment exceeding 15 days depressed induction in triticale anther culture. This may have been due to genotype effects, as we also observed reduced induction with some of the cultivars when cold pretreatment was prolonged from 2 to 3 weeks. Green plant production was, however, generally improved. In addition, prolonged cold pretreatment had a positive effect on spontaneous doubling [9]. Indrianto et al. [21] made a similar observation in wheat microspore culture. They discussed various alternatives for explaining this phenomenon: the effect of the anther wall, cold pretreatment per se or combination of stress treatments. These results indicate that cold stress is the decisive factor for increasing spontaneous doubling.

Aneuploidy has been detected in androgenetic plants of wheat [22], barley [23] and triticale [24]. Grass-like morphology was observed by Mix et al. [23] among barley DH-progeny, which were predominantly haploid, and by Hu and Kasha [22], who reported variable chromosome numbers (42 and 41) in wheat DH variants. In the flow-cytometric analyses the average DNA-content was highest for those plants classified as normal and lowest for those classified as stunted (Table 5). We did not detect any mixoploids in the analysis as reported by Mix et al. [23]. Among the 31 plants for which chromosome numbers were counted, five haploid plants were later classified as stunted. One of them had 19 and another 20 chromosomes, and only one had the expected 21 chromosomes in all cells observed. Charmet et al. [24] reported that the anomalies in triticale DHs were mainly whole chromosome variations among the rye genome. The distinct morphological changes observed here, and also in other anther culture progeny (S. Immonen, unpublished data), could be caused by a particular chromosome having a higher probability to be lost than others, as was observed by Gustafson and Zillinsky [25] for the rye chromosomes in triticale. They observed that the largest rye chromosome, 2R, was the first to be lost, while the smallest, 1R, was present in all plants analysed. However, Charmet et al. [24] noted that chromosomes 5R, 1R and 3R, in that order, were mostly involved in the variation. Charmet et al. [24] studied DH-progeny of F1 generations, where meiotic irregularities are common, and proposed that microspore anomaly was the cause of the chromosome alterations. In our study the mother plants were adapted cultivars expected to undergo a stable meiosis. Therefore, the aneuploidy observed here may have been caused by gametoclonal variation. Morphologically detectable

### Table 4

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>n</th>
<th>2C (%)</th>
<th>±</th>
<th>Confidence limits (95%)</th>
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<tbody>
<tr>
<td>LP 4496.5.92</td>
<td>15</td>
<td>66.7</td>
<td>24.1</td>
<td>42.6</td>
</tr>
<tr>
<td>‘Modul’</td>
<td>19</td>
<td>63.6</td>
<td>21.9</td>
<td>41.7</td>
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<tr>
<td>‘OAC Wintri’</td>
<td>40</td>
<td>55.0</td>
<td>15.6</td>
<td>39.4</td>
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<tr>
<td>‘Paljus’</td>
<td>83</td>
<td>32.5</td>
<td>10.2</td>
<td>22.3</td>
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<tr>
<td>‘OAC Trillium’</td>
<td>31</td>
<td>41.9</td>
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<tr>
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<td>16</td>
<td>37.5</td>
<td>24.0</td>
<td>13.5</td>
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<tr>
<td>‘Ulrika’</td>
<td>17</td>
<td>23.5</td>
<td>20.4</td>
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<tr>
<td>‘Asmus’</td>
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<tr>
<td>Bor 96151</td>
<td>85</td>
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Table 5

<table>
<thead>
<tr>
<th>Ploidy</th>
<th>Morphology</th>
<th>% (n)</th>
<th>DNA content pg (plants)</th>
<th>Fertile spikes</th>
<th>Seeds/fertile spike</th>
<th>Seeds/plant</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&gt;10 seeds</td>
<td>&lt;10 seeds</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Normal</td>
<td>75.2 (167)</td>
<td>19.5 (126)</td>
<td>0/7</td>
<td>7/7</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>Stunted</td>
<td>5.9 (13)</td>
<td>18.9 (7)</td>
<td>0/0</td>
<td>0/0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Grass</td>
<td>18.9 (42)</td>
<td>19.2 (26)</td>
<td>0/4</td>
<td>4/4</td>
<td>2.5</td>
</tr>
<tr>
<td>2C</td>
<td>Normal</td>
<td>73.4 (70)</td>
<td>35.7 (61)</td>
<td>153/192</td>
<td>39/192</td>
<td>20.4</td>
</tr>
<tr>
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<td>Stunted</td>
<td>12.6 (12)</td>
<td>34.6 (9)</td>
<td>1/8</td>
<td>7/8</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>Grass</td>
<td>13.7 (13)</td>
<td>34.9 (8)</td>
<td>2/7</td>
<td>5/7</td>
<td>4.0</td>
</tr>
</tbody>
</table>

Aneuploidy was considerably less than the aneuploidy observed by Charmet et al. [24] (23 vs 56%, respectively). Triticale may tolerate less aneuploidy in the haploid state, which would explain why there were proportionally fewer stunted plants in the haploid category than in the 2C category. The high proportion of grass-like plants among the haploid group may be due to difficulties in distinguishing aneuploidy-related morphology from the slenderness resulting from the haploid state. We observed individual seeds in some plants, which according to the initial analysis had been classified as haploids. Such plants were reported by Mix et al. [23] who found low percentages of diploid cells in their root tips. In this study flow-cytometry did not reveal such low levels of mixoploidy, and it is possible that the ratio of doubled cells increased during the vernalisation period. Among the normal 2C plants four were completely sterile. For two of them extreme DNA contents were recorded (1.9 pg below and 2.5 pg above the mean DNA content). Sterility in these cases seems to have been caused by chromosomal abnormality.

It seems possible to improve the androgenetic response of triticale through stress treatments, although stress may not be sufficient to induce androgenetic response in the most recalcitrant genotypes. Aneuploidy seems to be relatively common in the first DH generation of triticale. In practical triticale breeding, application of DH-technology at a more advanced generation than the F1, as suggested by Charmet et al. [24], could assist in reducing aneuploidy. On the other hand, aneuploid series could be exploited for scientific purposes [2]. However, in breeding applications aneuploidy seems to still represent a problem with triticale.

Acknowledgements

We thank Boreal Plant Breeding for providing seeds of the triticale cultivars. Marja-Leena Manninen and Iina Merjankari are gratefully acknowledged for their excellent technical assistance. Airi Tauriainen and Leena Ramstedt are thanked for their assistance in the flow-cytometric analysis.

References


